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Interaction of thioredoxin with oxidized aminobutyrate aminotransferase

Evidence for the formation of a covalent intermediate

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Pig brain 4-aminobutyrate aminotransferase is inactivated by pre-incubation with pyrroloquinoline quinone (2,7,9-tricarboxy-1H-pyrrolo[2,3,f]quinoline-4,5-dione; PQQ) at pH 7. The reaction of approximately 2 SH residues/dimer is sufficient to inactivate the enzyme. Reoxidized aminotransferase is reactivated by *E. coli* thioredoxin. Similar results were obtained with *E. coli* 4-aminobutyrate aminotransferase. The spectroscopic properties of thioredoxin, tagged with the fluorescence probe, anthraniloyl, were used to monitor its interaction with re-oxidized 4-aminobutyrate aminotransferase. During the regeneration of native aminotransferase by thioredoxin, the substrate forms a covalent intermediate with the oxireductase, as revealed by gel filtration chromatography. It is postulated that the substrate (oxidized aminotransferase) forms a covalent intermediate with thioredoxin through disulfide linkages.

Aminotransferase; Disulfide exchange; Thioredoxin; Fluorescence; Covalent intermediate; Protein interaction

1. INTRODUCTION

4-Aminobutyrate aminotransferase is a dimeric protein made up of subunits of identical molecular weight [1,2]. The enzyme catalyzes the transamination of 4-aminobutyrate to yield succinic semialdehyde and bound pyridoxamine-5-P. The cofactor, pyridoxal-5-P, is regenerated after transamination with 2-oxoglutarate. Oxidation of SH groups of the protein leads to the formation of disulfide bonds crosslinking the two subunits of the mitochondrial enzyme [3]. The enzyme species containing the disulfide bond are catalytically inactive, but they recover catalytic activity upon reduction of the disulfide bonds. Since the recovery of catalytic activity depends only on the presence of thiol compounds which are engaged in disulfide-thiol exchange reactions, it was thought of interest to investigate whether thioredoxin acts as a reducing agent of oxidized 4-aminobutyrate aminotransferase. Experimental evidence supporting the formation of a covalent intermediate between oxidized 4-aminobutyrate aminotransferase and thioredoxin is presented.

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Abbreviations: PQQ, pyrroloquinoline quinone; DTNB, 5,5'-dithio bis-(2-nitrobenzoic acid).

2. EXPERIMENTAL

2.1. *Purification of enzymes*

Mitochondrial 4-aminobutyrate aminotransferase was purified from pig brain according to a procedure previously described [1]. The preparation has a specific activity of 20 U/mg at 25°C, and it migrates as a single protein band on polyacrylamide gel electrophoresis. 4-Aminobutyrate aminotransferase from bacteria (*E. coli*, Y1090 strain) was purified by a procedure similar to that described for the purification of the enzyme from *Pseudomonas* [4]. After successive separations using DEAE–cellulose, hydroxyapatite and Sephacryl-S-300 chromatography, a purified protein of 110 kDa was obtained. The specific activity was 10 U/mg of protein at 25°C. Thioredoxin from *E. coli*, purified from overproducing clones [5], was a gift from Dr. Young Tae Kim. The concentration of purified thioredoxin was determined using an extinction coeffient ε_{280} of 13,700 $M^{-1} \cdot cm^{-1}$ for a molecular weight of 11.6 kDa [5].

Reduction of thioredoxin was achieved by incubating the protein (4 mg/0.2 ml) with 10 mM dithiothreitol (DTT) at pH 7 in 50 mM potassium phosphate buffer. Excess of reducing agent was removed by filtration through a Sephadex-G-25 column (10 × 1 cm) equilibrated with 100 mM potassium phosphate (pH 5.9). Reduced thioredoxin was immediately used in the reactivation of oxidized 4-aminobutyrate aminotransferase.

2.2. *Enzyme assays*

A coupled assay system consisting of two enzymes, i.e. 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase, was used to study the catalytic conversion of 4-aminobutyrate to succinic semialdehyde [1]. Enzymatic assays were performed in 0.1 M potassium phosphate (pH 8.4) containing 5 mM NAD^+, 30 mM 4-aminobutyrate and 10 mM 2-oxoglutarate. Changes in absorbance were monitored at 340 nm for at least 2 min.

Oxidoreductase activity of thioredoxin was determined by the turbimetric method [6] using solutions of insulin (1 mg/ml) in 0.1 M potassium phosphate (pH 7) and 0.3 mM DTT. Absorbance was measured at 650 nm.

2.3. *Labeling of thioredoxin*

Oxidized thioredoxin, 2 mg/ml, was allowed to react with a 2-fold molar excess of isatoic anhydride at pH 7.5 in 0.1 M potassium phosphate, 37°C. The reaction was allowed to proceed for 3 h, and excess of isatoic anhydride was removed by gel filtration through a Sephadex-G-25 column (10 × 1 cm) equilibrated with 100 mM potassium phosphate (pH 7). The degree of labeling was determined spectrophotometrically using an extinction coefficient of 4.6×10^3 $M^{-1} \cdot cm^{-1}$ at 330 nm. Anthraniloyl thioredoxin was reduced with DTT (10 mM) and passed through a Sephadex-G-25 column prior to the reconstitution studies. Concentration of free sulphydril groups was determined spectrophotometrically at 420 nm using DTNB (5,5'-dithio-bis(2-nitrobenzoic acid) [7].

2.4. *Spectroscopy*

Absorption spectra were recorded in a Shimadzu UV-160 spectrophotometer. Fluorescence measurements were performed in a SLM apparatus. Fluorescence decay measurements were made using the monophotonic technique with an Ortec nanosecond spectrometer. Excitation was set up at 330 nm and the emission passed through a Corning filter (C.S.3-74). The fluorescence decay curves were accurately fitted to either mono- or bi-exponential decay using non-linear least squares analysis [8].

2.5. *Materials*

Cysteamine, DTT, NAD^+ and insulin were purchased from Sigma; DTNB and isatoic anhydride from Aldrich; chromatographic materials, i.e. Sephacryl-S-300 and Sephadex-G-25 from Pharmacia, and DEAE-cellulose from Whatman.

3. RESULTS

Like other SH-containing proteins, 4-aminobutyrate aminotransferase is inactivated by pyrroloquinoline quinone (2,7,9-tricarboxy-1H-pyrrolo[2,3,f]quinoline-4,5-dione; PQQ) [9], a mild oxidizing compound which is the cofactor of methanol and glucose dehydrogenase from bacteria [10]. Complete loss of catalytic activity is attained by incubating the enzyme (20 μM) with PQQ (0.2 mM) for 1 h at 25°C in 0.1 M potassium phosphate (pH 7.4).

To ascertain whether oxidation of SH groups had taken place, samples of PQQ-treated enzyme were passed through a Sephadex-G-25 column equilibrated with 50 mM potassium phosphate (pH 7) and titrated with DTNB in the presence of 4 M guanidine-HCl. The results of the spectrophotometric measurements indicate the oxidation of approximately 2 SH residues/enzyme dimer (Table I). Samples of oxidized 4-aminobutyrate aminotransferase were allowed to preincubate with the reducing agents, cysteamine, glutathione, and thioredoxin; and the recovery of catalytic activity monitored as described in section 2. As shown by the results included in Fig. 1, recovery of catalytic activity is attained in the presence of cysteamine (2 mM) and thioredoxin (10 μM), but a small recovery of the original catalytic activity was achieved upon addition of reduced glutathione (10 mM).

Judging from the time–course of recovery of catalytic activity, it appears that a concentration of thioredoxin at least 200-fold lower than that of cysteamine is needed for full recovery of catalytic activity. The reactivation process is accelerated by increasing the concentration of reduced thioredoxin in the pre-incubation mixtures. Indeed, 90% of the catalytic activity of the aminotransferase is achieved within 30 min at a molar mixing ratio of substrate/thioredoxin of 1:4.

Table I

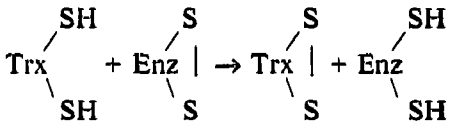
Inactivation of pig brain 4-aminobutyrate aminotransferase by PQQ

Sample	Activity[a] (%)	SH residues[b] (mol/dimer)
Aminotransferase	100	5.8
Aminotransferase + PQQ	2	4.0

[a]Samples of enzyme (20 μM) were incubated with PQQ (0.2 mM) for 1 h at 25°C in 0.1 M potassium phosphate buffer (pH 7.4). The samples were passed through a Sephadex-G-25 column to remove PQQ prior to enzymatic assays and determination of SH content.
[b]SH content determined in the presence of guanidinium-HCl (4 M).

3.1. *Mechanism of the reaction*

In view of the preceding results, it appears that reactivation of oxidized 4-aminobutyrate aminotransferase proceeds according to the following scheme:



This mechanism, which is identical to that originally proposed by Holmgren [6], does not take into account the formation of a covalent intermediate between the substrate and thioredoxin prior to the release of the products.

If thioredoxin acts by forming mixed disulfide bonds with the aminotransferase, then one should be able to show that thioredoxin is trapped by the aminotransferase during the reactivation process. Thioredoxin tagged with anthraniloyl is suitable for these experiments for the following reasons: (i) the reaction of isatoic anhydride with nucleophilic groups of the protein does not impair the function of thioredoxin, (ii) labeled thioredoxin activates oxidized 4-aminobutyrate aminotransferase in the manner depicted in Fig. 1, and (iii) the fluorescence properties of the anthraniloyl chromophore permit the detection of very low concentrations of the tagged protein. The spectroscopic properties are included in Fig. 2. 1 ml of a reaction mixture containing oxidized 4-aminobutyrate aminotransferase (10 μM) and anthraniloyl thioredoxin (reduced, 50 μM) in 50 mM potassium phosphate buffer (pH 7) was allowed to incubate at 25°C. Aliquots (0.2 ml) withdrawn at several time intervals were reacted with iodoacetate (10 mM) and applied to Sephacryl-S-300 columns.

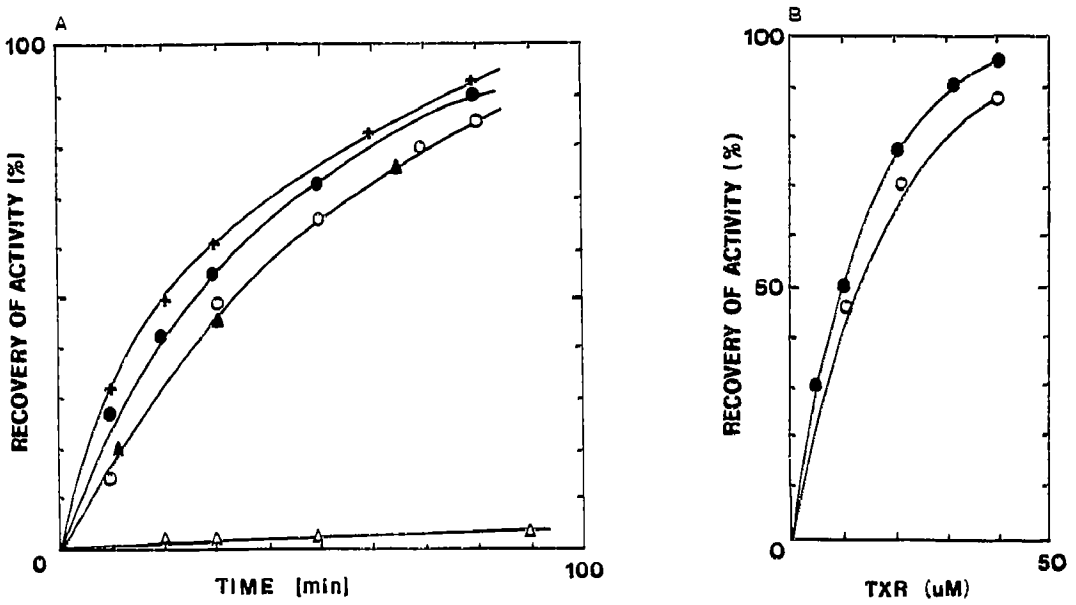


Fig. 1. (A) Time-course of reactivation of oxidized 4-aminobutyrate aminotransferase. Samples of oxidized enzyme (10 μM) were incubated with reduced thioredoxin (10 μM) in 50 mM potassium phosphate (pH 7) at 25°C. Aliquots withdrawn at indicated times were assayed for aminotransferase activity: results obtained with *E. coli* enzyme (+), and mammalian enzyme (●). Activation of the *E. coli* enzyme (▲) and mammalian enzyme (○) by cysteamine (2 mM). The effect of reduced glutathione (10 mM) on the recovery of the catalytic activity of oxidized pig brain 4-aminobutyrate aminotransferase (△) is included. (B) Effect of thioredoxin concentration. Samples of oxidized enzyme isolated from *E. coli* (10 μM) were incubated with increasing concentration of reduced thioredoxin (●) and anthraniloyl thioredoxin (○) in 50 mM potassium phosphate (pH 7) at 25°C for 30 min prior to enzymatic assays.

Fig. 3 shows the results obtained when the reaction mixture, pre-incubated for 5 min prior to addition of iodoacetate, was filtered through the gel filtration column. Two fluorescence peaks, characterized by different retention times are discernible in the elution pattern. The first elution peak has a retention time similar to 4-aminobutyrate aminotransferase (110 kDa), whereas the second elution peak is identical to free anthraniloyl thioredoxin. The fluorescence intensity corresponding to the first elution band is progressively decreased as the incubation time is prolonged. Thus, the elution profile of 4-aminobutyrate aminotransferase fully reactivated by anthraniloyl thioredoxin shows one peak; its elution retention time coincides with that of free anthraniloyl thioredoxin. The presence of a faster elution peak easily detected by fluorescence measurements could be assigned to the labeled protein of 12 kDa covalently linked to the unlabeled enzyme of 110 kDa.

4. DISCUSSION

The results presented in this work indicate an important role of thioredoxin in preventing the inactivation of 4-aminobutyrate aminotransferase in prokaryotes and eukaryotes. In this connection it is worth noting that bacterial, plant and animal cells contain thioredoxin, which has evolved from a common ancestor [11]. The redox protein of 12 kDa is widely distributed in subcellular fractions, i.e. nuclei, microsomes, and mitochondria of mammalian cells [12]. Therefore, it was not surprising to find that thioredoxin from *E. coli* is efficient in catalyzing thiol–disulfide exchange reactions in a protein isolated from bacteria and mammalian cells. An interesting feature of our studies is the finding that during reduction of the aminotransferase, a covalent intermediate could be detected by monitoring the emission properties of protein fractions eluted from a gel filtration column. Thioredoxin labeled with a fluorescent probe (anthraniloyl) could be used to assess the presence of macromolecular intermediates in reactions which are known to be catalyzed by the redox protein. As an example, the activation of phosphoribulose ki-

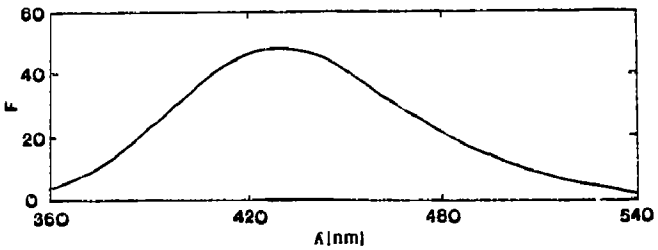


Fig. 2. Emission spectra of anthraniloyl thioredoxin excited at 330 nm. The protein conjugate contains 1 mol of anthraniloyl/mol of thioredoxin. The fluorescence quantum yield of the bound chromophore is 0.12 and decay time $\langle\tau\rangle$ = 10.7 ns.

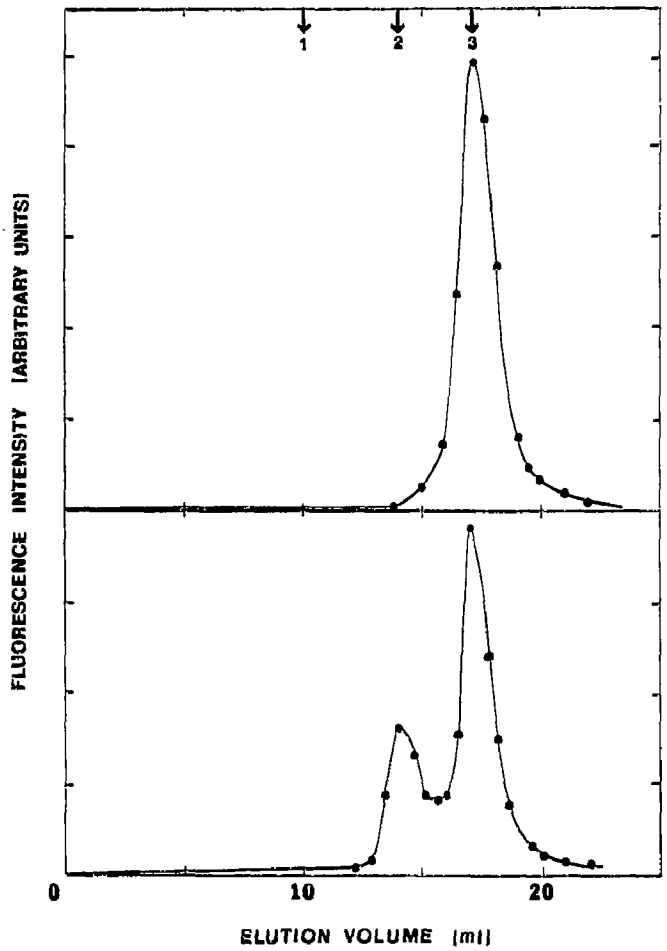


Fig. 3. Elution profiles from gel filtration monitored by fluorescence measurements. 1 ml of a reaction mixture containing oxidized 4-aminobutyrate aminotransferase (10 μM) and anthraniloyl thioredoxin (50 μM) in 50 mM potassium phosphate (pH 7) was allowed to incubate at 25°C. Aliquots withdrawn (0.2 ml) after 5 and 30 min incubation were allowed to react with iodoacetate (10 mM) and applied to a Sephacryl-S-300 column (34 ml, total volume). (Bottom) Results obtained with the reaction mixture pre-incubated for 5 min prior to addition of iodoacetate. (Top) Results obtained with the sample incubated for 30 min. Emission measured at 420 nm upon excitation at 330 nm. The void volume (1), and the elution of 4-aminobutyrate aminotransferase (2) and thioredoxin (3) are indicated.

nase by thioredoxin f is related to the reduction of a disulfide bond in the kinase [13]. Apparently, the sensitivity of the kinase towards thioredoxin depends on the degree of accessibility of a disulfide bond formed between Cys^{16} and Cys^{55} [14]. Although this disulfide bond is the site of reduction by thioredoxin f, there is no information available on the mechanism of the reaction.

Finally, it should be noted that Hu and Tson [15] have reported that protein disulfide isomerase (PDI) interacts with scrambled RNAse through the formation of intermolecular disulfide linkages. Since the function of PDI and thioredoxin is to catalyze inter- and intramolecular thiol–disulfide exchange reactions, it seems reasonable to suggest that the reactions catalyzed by both proteins proceed through the same mechanistic steps.

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REFERENCES

[1] Churchich, J.E. and Moses, U. (1981) J. Biol. Chem. 256, 1101–1104.
[2] Kwon, O.S., Park, J. and Churchich, J.E. (1992) J. Biol. Chem. 267, 7215–7216.
[3] Kim, D.S. and Churchich, J.E. (1987) J. Biol. Chem. 262, 14250–14254.
[4] Yonaha, K. and Toyama, S. (1980) Arch. Biochem. Biophys. 200, 156–164.
[5] Tabor, S., Huber, H.E. and Richardson, C.C. (1987) J. Biol. Chem. 262, 16212–16223.
[6] Holmgren, A. (1979) J. Biol. Chem. 254, 9627–9632.
[7] Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70–77.
[8] O'Connor, D.V. and Phillips, D. (1984) Time-correlated Single Photon Counting, Academic Press, London.
[9] Park, J. and Churchich, J.E. (1992) Biofactors 3, 257–260.
[10] Duine, J.A., Frank, J. and Jongejan, J.A. (1989) Adv. Enzymol. 59, 170–209.
[11] Hall, D.E., Baldesten, A., Holmgren, A. and Reichard, P. (1971) Eur. J. Biochem. 23, 328–335.
[12] Holmgren, A. and Luthman, M. (1978) Biochemistry 17, 4071–4077.
[13] Buchanan, B.B. (1980) Annu. Rev. Plant Physiol. 31, 341–374.
[14] Porter, M.A., Stringer, C.D. and Hartman, F.C. (1988) J. Biol. Chem. 263, 123–128.
[15] Hu, C.H. and Tsou, C.L. (1991) FEBS Lett. 290, 87–89.